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Two-step recovery process for tryptophan tagged cutinase: Interfacing aqueous two-phase extraction and hydrophobic interaction chromatography

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Abstract

In this work, the interfacing of a poly(ethylene glycol) (PEG)–phosphate aqueous two-phase system with hydrophobic interaction chromatography (HIC) for primary recovery of an intracellular protein was evaluated. As a model protein, a recombinant cutinase furnished with a tryptophan–proline (WP) peptide tag was used and produced intracellularly in *Escherichia coli* (*E. coli*). *E. coli* cell homogenate was partitioned in a two-phase system and the top phase yield, concentration and purity of the tagged ZZ-cutinase-$(WP)_4$ was evaluated as function of polymer sizes, system pH and phase volume ratio. The partition behaviour of cell debris, total protein and endotoxin was also monitored. In the HIC part, the chromatographic yield and purity was investigated with respect to ligand hydrophobicity, dilution of loaded top phase and elution conditions. Based on the results, a recovery process was demonstrated where a PEG 1500-K–Na phosphate salt aqueous two-phase system was interfaced with a HIC column. The interfacing was facilitated by the Trp-tagged peptide. The tagged ZZ-cutinase-$(WP)_4$ was obtained in a PEG-free phase and purified to >95% purity according to silver stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels with a total yield of 83% during the two-step recovery process.
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1. Introduction

Aqueous two-phase systems have for a long time been used as a preparative method in biochemistry [1]. An aqueous two-phase system (ATPS) is formed when two incompatible polymers or a polymer and salt is mixed above a certain concentration. Proteins can then be partitioned between the phases and purified to a good extent. An ATPS can handle insoluble matter and is also easy to scale up [2–4]. Thus, this technique is well suited for applying as a primary recovery step in a protein manufacturing process, especially when working with large volumes containing high concentrations of particulate matter such as cells and cell debris.

An ATPS should not only have the ability to give a high yield of target protein, remove cells, cell debris and nucleic acids but should also be suitable for simple interfacing with purification operations downstream. Different tools can be used in making the purification process more efficient. The surface properties of a protein can be modified by genetic engineering through the addition of, e.g. different forms of peptide tags. This has proven to be successful in earlier work with aqueous two-phase systems [5,6]. By adding a tryptophan–proline (WP) tag to the target protein, the protein can be efficiently extracted into the top phase in poly(ethylene glycol) (PEG)/salt and polymer/polymer systems. The addition of such hydrophobic tags has also been proven to be efficient in hydrophobic interaction chromatography (HIC) [7,8] where the proteins can be eluted with different retention times. This is a more cost-efficient chromatographic method

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for protein purification since HIC is less expensive compared to affinity chromatography based on ligands such as immobilised metal affinity chromatography (IMAC), protein A and IgG [9–11].

In this work, a genetically engineered cutinase (ZZ-cutinase-wt (wild type) and ZZ-cutinase-$(WP)_4$) were used as model proteins. It was demonstrated that the physical modification of the target protein could be utilised in the design of a recovery process based on interfacing of aqueous two-phase extraction with HIC. The Z domain is derived from the B domain of staphylococcal protein A [5]. The purification tag $(WP)_4$ consists of four prolines and four tryptophans. In the ATPS part of the study *E. coli* cell homogenate (25 g dry weight/l) was partitioned and the top phase yield, concentration and purity of the tagged ZZ-cutinase-$(WP)_4$ was evaluated as a function of PEG polymer sizes, system pH and phase volume ratio. The partition behaviour of cell debris and endotoxin was also monitored. In the HIC part, PEG-rich top phases from the ATPS were loaded and the chromatographic yield and purity were investigated with respect to ligand hydrophobicity, dilution of loaded top phase and elution conditions. Based on the results, a primary recovery process starting with *E. coli* cell homogenate was demonstrated on laboratory scale where a PEG 1500-K–Na phosphate ATPS was interfaced with a Phenyl Sepharose HP HIC column (GE Healthcare).

2. Materials and methods

2.1. Model protein

The lipolytic enzyme cutinase from *Fusarium solani pisi* that hydrolyses soluble esters and triacylglycerols [12] was used as a model protein in this study. Two Z domains derived from the B domain of staphylococcal protein A [13,14] were previously fused to the N terminal of cutinase in order to facilitate the purification of the fusion protein by IgG affinity chromatography [5]. A hydrophobic tag consisting of four prolines and four tryptophans, $(WP)_4$, was fused to the C terminal of cutinase. The hydrophobically modified protein is further denoted as ZZ-cutinase-$(WP)_4$ whereas the protein with no fused C terminal tag is denoted as ZZ-cutinase-wt [5].

2.2. Protein production

The proteins were produced as a periplasmic product in kanamycin-resistant *Escherichia coli* (*E. coli*) strain MC 4100 containing the plasmid pK4ZZ-cutinase-$(WP)_4$ or pK4ZZ-cutinase-wt coding for ZZ-cutinase-$(WP)_4$ and ZZ-cutinase-wt, respectively [5]. The ZZ-cutinase-$(WP)_4$ and ZZ-cutinase-wt was grown in minimal salt medium containing (per litre): 1.6 g KH_2PO_4, 5.0 g $(NH_4)_2SO_4$, 6.6 g $Na_2HPO_4{\cdot}2H_2O$, 0.5 g di-ammonium hydrogen citrate. After medium sterilisation (autoclaving at 121 °C), 1 M $MgSO_4$ and 1 ml l^{-1} of a trace element solution was added by sterile filtration. The cells were cultivated and harvested under similar conditions described earlier [2]. The cells were stored at −80 °C.

2.3. Cell disruption

The harvested cells containing ZZ-cutinase-$(WP)_4$ and ZZ-cutinase-wt were diluted to 100 g l^{-1} dry weight (DW) and disintegrated in a French Pressure Cell Press from SLM Instruments Inc. (Rochester, USA) at 1.1×10^5 kPa for two rounds. The cells were then used for extraction in the two-phase system without further treatment.

2.4. Aqueous two-phase partitioning

The phase-forming chemicals PEG 1500 (Mr = 1400–1600 g/mol), PEG 4000 (Mr = 3500–4000 g/mol), K_2HPO_4 and NaH_2PO_4 were purchased from Merck (Darmstadt, Germany). Both PEG and the K_2HPO_4/NaH_2PO_4 salt (further denoted in the text as K–Na phosphate salt) were prepared as 40% weight/weight (w/w) stock solutions. Different base/acid molar ratios of K_2HPO_4/NaH_2PO_4, i.e. 15, 20, 40 and ∞ were prepared and tested in the systems. Two-phase systems with a final weight of 10 g were made up from PEG, K–Na phosphate salt, and homogenate in 15 ml graduated tubes. The cell homogenate was added to the system to a final weight of 25 g l^{-1} dw. All systems were made in duplicate and blank systems devoid of homogenate were also prepared. The systems were then mixed carefully and placed in a water bath at 25 °C for 20 min followed by centrifugation at 1500 rpm ($700 \times g$) for 5 min in a Wifug laboratory centrifuge (Great Norton Bradford, UK). The volume of the top and bottom phases was estimated and separated for further analysis and processing.

2.5. Analytical methods

The cutinase activity was determined by using a standard activity assay composed of mixed micelles, detergent and substrate as described earlier. The enzymatic activity, dC/dt (U/ml) was calculated by using an extinction coefficient of 15.4 cm^2 μmol^{-1} for *para*-nitrophenol. To minimise the interference of the phase chemicals in the system, each phase sample with enzyme was mixed with an equal volume of the opposite phase devoid of enzyme. All samples were diluted properly with assay buffer to achieve a homogenous sample.

The total protein concentration in the samples was determined with the BCA protein assay method according to the manufacturer's manual Pierce (Rockford, USA). Each sample was compensated for disturbing substances by using samples from blank systems devoid of proteins. The protein samples were also analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SDS–PAGE, on homogenous 12% NuPage Bis–Tris pre-cast gels (Invitro-

gen, Carlsbad, Germany) under reducing conditions with 50 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS) Merck (Stockholm, Sweden). Electrophoresis conditions were set to 200 V, 125 mA for 60 min. The gels were stained in either Coomassie Brilliant Blue R 250 GE Healthcare (Uppsala, Sweden) and destained in water or silver stained according to [15,16].

The concentration of PEG in the samples was determined by using the method described earlier by Skoog [17]. The endotoxin was analysed using a *Limulus* amebocyte lysate test kit, Associates of Cape Cod (Falmouth, MA, USA).

2.6. Calculations

The target protein partitioning in an aqueous two-phase system is described by the partition coefficient K, and defined as $K = C_T/C_B$ where C_T and C_B correspond to the concentrations in the top and bottom phases, respectively. The yield (%) of the target protein in the top phase is calculated as:

$$Y = \frac{C_T^{\text{Cut}} \times V_T}{C_0^{\text{Cut}} \times V_0} \times 100 \quad (1)$$

where C_T^{Cut} and C_0^{Cut} are the concentrations of cutinase (U/ml) in the top phase and in the homogenate, respectively, and V_T and V_0 are the volumes of the top phase and of the homogenate, respectively.

The purification factor of cutinase in the aqueous two-phase system is defined as:

$$\text{PF} = \frac{C_T^{\text{Cut}} \times C_0^{\text{TOT PROT}}}{C_T^{\text{TOT PROT}} \times C_0^{\text{Cut}}} \quad (2)$$

where $C_T^{\text{TOT PROT}}$ and $C_0^{\text{TOT PROT}}$ are the total protein concentrations in the top phase and in the homogenate, respectively.

2.7. Hydrophobic interaction chromatography (HIC)

The top phase was further purified at room temperature on a FPLC system GE Healthcare (Uppsala, Sweden). A HiTrap HIC selection kit (GE Healthcare) was used for screening tests containing five 1 ml columns, Phenyl FF (high substitute (sub)), Phenyl FF (low sub), Phenyl HP, Butyl FF and Octyl FF. For the screening tests the protocol described in Table 1 was used. The absorbance of the proteins was monitored with an UV detector, UV-MII. One-millilitre fractions were collected with a Fraction collector 100 (GE Healthcare) and the enzyme activity was measured over the entire chromatogram.

2.8. Calculations for hydrophobic interaction chromatography

The yield and the purification factor for the hydrophobic interaction chromatography and the overall yield in the two-step purification process were calculated. The yield in the HIC step and the overall yield were calculated as:

Table 1
Chromatographic parameters for screening of the different HIC columns for purification of ZZ-cutinase-$(WP)_4$ from a PEG top-phase

Column volumes (ml)	Buffer	Step	Flow rate (ml/min)
5	Running buffer	Equilibration of column	1
5	Running buffer	Sample load	0.5
10	Running buffer	Wash	1
20	100% Elution buffer	Elution	1
10	Running buffer	Wash	1

As running buffer a 347 mM K_2HPO_4/NaH_2PO_4, mole ratio 15, pH 8.3 was used. The sample was eluted in 50 mM sodium phosphate buffer (pH 7), if not otherwise stated.

$$Y_{\text{HIC}} = \frac{C_{\text{HIC Peak}}^{\text{Cut}} \times V_{\text{HIC Peak}}}{C_T^{\text{Cut}} \times V_{T\,\text{add}}} \times 100 \quad (3)$$

$$Y_{\text{TOT}} = \frac{C_{\text{HIC Peak}}^{\text{Cut}} \times V_{\text{HIC Peak}}}{C_0^{\text{Cut}} \times V_0} \times 100 \quad (4)$$

where $C_{\text{HIC Peak}}^{\text{Cut}}$ is the concentration of ZZ-cutinase-$(WP)_4$ in the HIC peak. $V_{T\,\text{add}}$ and $V_{\text{HIC Peak}}$ are the volumes of top phase added to the column and of the eluted HIC peak from the column, respectively.

The purification step of cutinase in the HIC step and the overall two-step purification process is calculated as:

$$\text{PF}_{\text{HIC}} = \frac{C_{\text{HIC Peak}}^{\text{Cut}} \times C_T^{\text{TOT PROT}}}{C_{\text{HIC Peak}}^{\text{TOT PROT}} \times C_T^{\text{Cut}}} \quad (5)$$

$$\text{PF}_{\text{TOT}} = \frac{C_{\text{HIC Peak}}^{\text{Cut}} \times C_0^{\text{TOT PROT}}}{C_0^{\text{Cut}} \times C_{\text{HIC Peak}}^{\text{TOT PROT}}} \quad (6)$$

where the $C_{\text{HIC Peak}}^{\text{TOT PROT}}$ are the total protein concentrations of the ZZ-cutinase-$(WP)_4$ in the top phase and the peak eluted from the HIC column, respectively.

3. Results and discussion

The hydrophilic salt forming the bottom phase have earlier in PEG/salt systems mainly been composed of potassium phosphate [3,18]. In this work, we have used a base/acid salt pair composed of K_2HPO_4/NaH_2PO_4 (K–Na phosphate salt) due to the higher solubility of this pair compared to Na_2HPO_4/KH_2PO_4 or to a pair with the same cation. The higher solubility can be useful in industrial applications since higher concentrations of stock solutions can be used. Furthermore, earlier results have shown that different mole ratios of the K–Na phosphate salt did not affect the position of the binodial curve compared to when a base/acid salt pair with the same counter ion is used [19]. In order to design a primary recovery step for purification of recombinant protein different parameters such as polymer concentration, molecular weight of the polymers and pH can be varied to influ-

ence the target proteins partitioning in the two-phase system [1,20,21].

3.1. Impact of cells and cell debris on the two-phase system

In a primary recovery step when a homogenised sample containing cellular components is added to the ATPS, it is desirable to recover the target protein in the top phase and simultaneously remove cells and cell debris to the bottom phase. However, the cells and cell debris partitioned mainly to the interface in our systems. In technical applications when continuous centrifugal separators are used this might not be the most optimal situation affecting the efficiency of the operation negatively. Thus, there could be need for improvement with respect to the partition behaviour of particulate matter in the cutinase extraction. One approach could be to change the *E. coli* host cell/strain used for cutinase expression. Thus, in the case where a different strain was used β-galactosidase was extracted to the top PEG phase in a PEG/salt system while the cells and cell debris could be recovered in the bottom salt phase [22].

3.2. Partitioning of ZZ-cutinase-$(WP)_4$ in PEG/K–Na systems

3.2.1. Influence of PEG molecular weight and pH of the systems

To test if the molecular weight of the PEG polymer and pH of the system had any influence on the *K*-value and yield of the target protein different base to acid mole ratios (K/Na), 10, 15, 20, 40 and ∞ were tested in two different systems composed of 10% PEG 4000/9.4% K–Na phosphate salt and 14.8% PEG 1500/12.3% K–Na phosphate salt. It was observed (Fig. 1) that the different mole ratios of the salt did not have any large influence on *K* in either system. However, the yield of the ZZ-cutinase-$(WP)_4$ increased when a mole ratio above 10 was used in systems composed of both PEG 1500 and PEG 4000. The yield has an optimum for mole ratios between 15 and 40 in systems composed of the PEG 1500 polymer, and above a mole ratio of 40 the yield starts to decrease. The yield in systems composed of PEG 4000 varies more with the mole ratio compared to the PEG 1500 systems. The highest yield is obtained when a mole ratio of 15 or 40 is used. A decrease in yield is observed at a mole ratio of 20 whereas this is not observed in systems composed of PEG 1500. The results (Fig. 1) also show that the yield of the target protein was higher in a system containing the PEG 1500 polymer compared to systems containing the PEG 4000 polymer. Based on the above results, it was decided to further study systems composed of PEG (Mw 1500 and 4000) and K–Na phosphate salt with a mole ratio of 15.

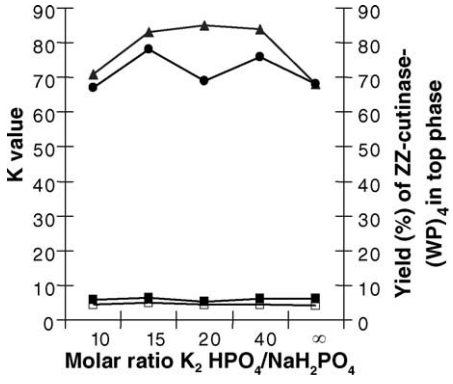

Fig. 1. The *K* and yield of ZZ-cutinase-$(WP)_4$ after extraction in aqueous two-phase systems composed of either PEG 1500 or PEG 4000 and different mole ratios of K_2HPO_4/NaH_2PO_4. The systems were composed of 10% (w/w) PEG 4000 and 9.4% (w/w) K–Na phosphate salt and 14.8% (w/w) PEG 1500 and 12.3% (w/w) K–Na phosphate salt. (■) *K*-value for PEG 1500; (▲) yield (%) for PEG 1500; (□) *K*-value for PEG 4000 and (●) yield (%) for PEG 4000.

3.2.2. Influence of tie-line length

Another parameter to vary in order to increase the recovery in the two-phase system is the polymer and salt concentration. It has earlier been shown that by increasing or decreasing the tie-line length a more extreme partitioning of the target protein could be observed to either the top or bottom phase [1,21,23]. Thus, different tie-line lengths were tested by keeping the PEG 4000 and PEG 1500 polymer concentration constant (10% and 14.8%) and decreasing and increasing the K–Na phosphate salt concentrations. From the experiments (Table 2), it can be concluded that in PEG 4000 systems, the *K*-value of ZZ-cutinase-$(WP)_4$ increased with increasing tie-line length. The system composed of 10% PEG 4000 and 12% K–Na phosphate salt seems to be the best operating point with respect to *K*-value for the systems composed of the PEG 4000 polymer (Table 2). Between the two systems with highest *K*-value (4.9 and 6.2), the system with the shorter tie-line length (lower concentration of phase-forming chemicals) is more desirable for economical reasons from a scale up point of view. Thus, the system composed of 10% PEG 4000 and 9.4% K–Na phosphate salt was selected for further studies.

The results for PEG 1500 systems composed of different concentrations of K–Na phosphate salt (Table 3) showed that an increased tie-line length lowered the *K*-value of ZZ-cutinase-$(WP)_4$, whereas a decrease in tie-line length increased the *K*-value compared with the starting operating composition (14.8% PEG 1500 and 12.3% K–Na phosphate

Table 2
Partitioning (*K*), yield (*Y*) and volume ratio (V_T/V_B) of ZZ-cutinase-$(WP)_4$ from *E. coli* homogenate (25 g/l DW) in aqueous two-phase systems with different tie-line lengths

Salt concentration (w/w) (%)	V_T/V_B	*K*	Yield (%) in TP
8.5	1.1	1.9	68
9.0	0.7	3.2	78
9.4	0.6	4.9	78
12.0	0.5	6.2	78

The PEG 4000 concentration is 10% (w/w) in all systems and the mole ratio of the K_2HPO_4/Na_2HPO_4 is 15.

Table 3
Partitioning (K), yield (Y) in top phase and volume ratio (V_T/V_B) of ZZ-cutinase-$(WP)_4$ from *E. coli* homogenate (25 g/l DW) in aqueous two-phase systems with different tie-line lengths

Salt concentration (w/w) (%)	V_T/V_B	K	Yield (%)
9	0.6	5.2	90
9.5	1.6	7.2	98
10.0	1.2	8.4	89
10.5	1.1	7.6	90
11.0	0.9	7.1	85
12.3	0.7	6.5	83
15.0	0.2	3.3	33

The PEG 1500 concentration is 14.8% (w/w) in all systems and the mole ratio of the K_2HPO_4/Na_2HPO_4 is 15.

salt). However, the K-value is not significantly different for the systems with K–Na phosphate salt concentrations in the range 9.5–12.3% (Table 3). The system composed of 14.8% PEG 1500 and 10.5% K–Na phosphate salt was chosen for further studies based on the high K-value but also since it is not positioned on a tie-line too close to the critical point where systems can be less robust causing large differences in K-value and yield.

3.2.3. Improvement of yield

In a primary recovery step, it is desirable to achieve both a high yield and a concentration of the target protein. In an ATPS, the yield and concentration of a target protein can be varied by moving along a tie-line changing the phase volume ratio (V_T/V_B). The phase compositions of the top and bottom phases are not changed during this procedure. Furthermore, in theory also, the K-value of, e.g. a target protein should remain unchanged [21]. If the top phase is the target phase, an increased phase volume ratio should theoretically increase the target protein yield, while the concentration of it should decrease. For the K-values obtained in our systems there is not room for concentration since phase volume ratios around 1 or larger seem to be needed in order to obtain yields around and above 90% (Tables 4 and 5). When phase volume ratios were altered along the tie-line including the system 10% PEG 4000 and 9.4% K–Na phosphate (Table 4) the results indicated that a smaller top phase resulted in a lower yield than for the starting composition (10% PEG 4000 and 9.4% K–Na phosphate) for systems composed of PEG 4000. A higher volume ratio, i.e. larger volume of the top phase should in theory result in a higher yield but this was not the case for the system composed of 12% PEG 4000 and 9% K–Na phosphate. Partly a reason for this was that the K-value of the target protein was not constant when operating along a tie-line, as stated by the theory [21]. Although a slight variation of K-values for different V_T/V_B was seen, it should be kept in mind that in a homogenate many substances, e.g. cells, cell debris and the rest of the broth medium components are present and can affect both the phase system and the partitioning.

Table 4
Partitioning (K), yield (Y) and volume ratio (V_T/V_B) of ZZ-cutinase-$(WP)_4$ from *E. coli* homogenate (25 g/l DW) in aqueous two-phase systems with different volume ratios and constant tie-line length

System composition (w/w) (%)		V_T/V_B	K	Yield (%) in TP
PEG 4000	K_2HPO_4/Na_2HPO_4			
8	10	0.4	5.2	73
10	9.4	0.6	4.9	78
12	9	1.2	4.1	72

The system is composed of PEG 4000/K–Na phosphate.

Table 5
Partitioning (K), yield (Y) and volume ratio (V_T/V_B) of ZZ-cutinase-$(WP)_4$ from *E. coli* homogenate (25 g/l DW) in aqueous two-phase systems with different volume ratios and constant tie-line length

System composition (w/w) (%)		V_T/V_B	K	Yield (%) in TP
PEG 1500	K_2HPO_4/Na_2HPO_4			
12.0	11.5	0.6	8.6	83
12.5	11.0	0.7	6.8	80
13.0	11.0	0.6	5.3	69
14.0	10.5	0.9	8.3	96

Different phase volume ratios along the same tie-line as where the 14.8% PEG and 10.5% K–Na phosphate salt system was positioned were also tested. It can be concluded (Table 5) that the system composed of 14% PEG 1500 and 10.5% K–Na phosphate resulted in high yield (95%), a high K (8.3) at a V_T/V_B of 0.9.

3.2.4. Conclusions for aqueous two-phase partitioning of ZZ-cutinase-$(WP)_4$

Based on the studies above it can be concluded that the partitioning of ZZ-cutinase-$(WP)_4$ was found to be best in a system composed of 14% PEG 1500 and 10.5% K–Na phosphate salt with a mole ratio of 15. The ZZ-cutinase-$(WP)_4$ could be recovered to 96% in the top phase with most of the particulate matter removed, which is desirable if packed bed chromatography is to be used for further purification downstream. The purification factor based on protein concentration meauserments in this system (14% PEG 1500 and 10.5% K–Na phosphate salt) is 2.5 compared to 6.4 as in the case for the system composed of 10% PEG 4000 and 9.4% K–Na phosphate salt. This can be explained by the lowering of the molecular weight of PEG polymer from 4000 to 1500. The lowering of the molecular weight increases the yield of the target protein in the top phase due to a higher mixing entropy. This higher mixing entropy leads to more favorable partitioning of the proteins to the top phase [24]. This effect was higher for the total proteins than for the ZZ-cutinase-$(WP)_4$, and therefore, the purification factor was decreased. The decrease in purification factor when decreasing the polymer molecular weight from 4000 to 1500 is in this case not a major issue since the top phase is being further purified by packed bed chromatography and is compensated by the high recovery of ZZ-cutinase-$(WP)_4$ (96%) in the top phase of PEG 1500.

3.3. Packed bed chromatography

There are a number of different ways of interfacing the top phase from the two-phase system with other techniques. If a protein is extracted in a thermoseparating polymer/polymer system, the protein will, after thermoseparation, be recovered in an almost polymer-free water phase [25,26] and is thus well suited for further downstream steps. In PEG/salt systems, a target protein that is recovered in the PEG phase can be back-extracted to a fresh salt phase in a subsequent extraction step and then interfaced with, e.g. membrane filtration or packed bed column chromatography [3]. However, membrane filtration is only suitable for rather large proteins (>100 kDa) [27]. If the target protein is recovered in the PEG phase in a-PEG/salt system, as in this work, the target protein will experience an environment with a PEG concentration of about 30%. The viscosity of such a phase has been reported to be in the range 9–16 mPa s, depending on the PEG molecular weight [22,28], which could cause problems with high pressure drops in packed bed chromatography. However, successful integration of two-phase systems with packed bed chromatography have been reported in earlier work [29]. Chymotrypsinogen B produced extracellular with *Pichia pastoris* was extracted in a PEG/salt system and then further purified on an ion exchange packed bed column (Sepharose Big Beads) with an overall recovery of 69%.

The use of hydrophobic interaction chromatography has been proven to be a suitable technique for purification of tryptophan and tyrosine tagged proteins [7,8]. Thus, the addition of tryptophan or tyrosin tags to a target protein might facilitate the interfacing of two separation steps that utilise the same physical property of the target protein. Furthermore, scale up of PEG/salt systems [4,22] and HIC [30] have proven to be successful and makes it attractive for industrial use.

3.3.1. Selection of proper running buffer

To select a proper running buffer for the chromatography, the buffer was set to resemble the conditions in the top phase. Thus, the salt concentration in the top phase was determined by measuring the conductivity in the PEG phase. A standard curve was conducted with conductivity for different concentrations of K–Na phosphate salt from a 40% stock solution. A top phase from a system composed of 14% PEG 1500 and 10.5% K–Na phosphate salt devoid of proteins was then diluted properly to determine the concentration of the salt in the PEG phase. The salt concentration in the top phase was determined to be 347 mM and the pH was 8.3. During the screening tests a running buffer with the above concentration and pH was used and the elution buffer was composed of 50 mM sodium phosphate, pH 7.

3.3.2. Screening of different HIC columns

To find a suitable HIC column for further purification of the top phase containing ZZ-cutinase-$(WP)_4$ a number of different columns were tested. A HIC selection kit containing five different columns, Butyl FF, Octyl FF, Phenyl FF (low substituted), Phenyl FF (high substituted) and Phenyl High Performance (HP) was used. To overcome the fact that the top phase contained both PEG and salt, which would create a two-phase system during purification on the column, the sample was diluted twice with running buffer during the screening tests. During the screening for the proper HIC column a similar chromatography protocol was used for all the runs (Table 1). It can be observed (Fig. 2) that ZZ-cutinase-$(WP)_4$ purified on a Butyl FF, Octyl FF and Phenyl FF (low sub) was eluted within the time of the protocol when eluted with 50 mM phosphate buffer. The Phenyl (high sub) had the longest retention time and was not eluted within the time of the protocol, indicating that this is the most hydrophobic column compared to Butyl FF, Octyl FF and Phenyl FF (low sub). The Phenyl HP was tested after the previously tested columns and eluted with 10 mM of phosphate buffer instead of 50 mM. The elution conditions were altered since it was suspected that the Phenyl HP column would give rise to a strong hydrophobic interaction with the ZZ-cutinase-$(WP)_4$, as in the case for Phenyl FF (high sub) (Fig. 2), and hence the

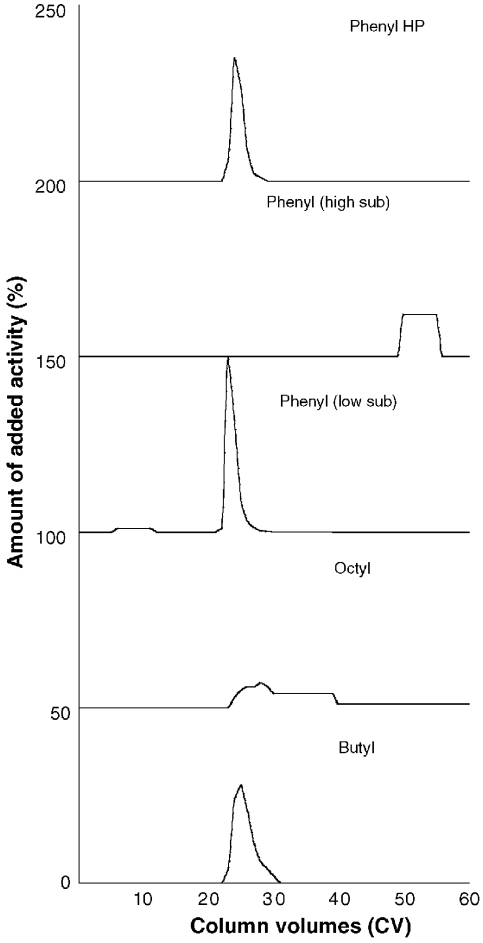

Fig. 2. Chromatogram for purification of ZZ-cutinase-$(WP)_4$ on Butyl FF, Octyl FF, Phenyl FF (low sub), Phenyl FF (high sub) and Phenyl HP. The scale on the *Y*-axis is divided between 0 and 50% indicating that every column after Butyl FF has its zero point at 50% of the added activity of the column before.

Table 6
The yield (%) of ZZ-cutinase-$(WP)_4$ purified from a top phase and mass balances for the different HIC columns used during the screening tests

	Butyl FF	Octyl FF	Phenyl FF (low sub)	Phenyl FF (high sub)	Phenyl HP
Yield (%)	100	69	91	77	78
Mass balance	100	74	100	78	85

protein would not elute within the protocol. The yield of the eluted peak and mass balance was calculated for the different HIC columns (Table 6). The columns with the highest eluted peak yields were Butyl FF and Phenyl FF (low sub). Both of these columns resulted in a mass balance close to 100%. The other columns, Octyl, Phenyl HP and Phenyl FF (high sub) had lower eluted peak yields and their mass balances did not close. However, the Phenyl HP and Phenyl FF (high sub) all eluted in a peak with a lower amount of contaminating proteins according to SDS–PAGE (results not shown). Furthermore, the Octyl FF and Butyl FF columns were eluted in relatively large volumes (32 ml and 18 ml) compared to the starting volume (1 ml) (Fig. 2). The selection criteria for further purification development were based on purity shown by SDS–PAGE (results not shown) and the dilution factors of the eluted peaks containing the target proteins. Hence, it was decided that Phenyl HP, Phenyl (low sub) and Phenyl (high sub) should be further evaluated.

3.3.3. Further development of HIC with Phenyl FF (low sub) and Phenyl FF (high sub)

The Phenyl FF (low sub) and Phenyl FF (high sub) were further tested for purification of the ZZ-cutinase-$(WP)_4$ protein. An undiluted sample was injected onto the column to investigate if the dilution of the sample containing ZZ-cutinase-$(WP)_4$ had any impact on the interaction with the Phenyl FF (low sub) and Phenyl FF (high sub). Furthermore, the concentration of the elution buffer was decreased to 10 mM (Phenyl (low sub)) and 5 mM (Phenyl (high sub)) sodium phosphate buffer. The lowering of elution buffer concentration decreases the hydrophobic interactions and the protein was eluted within the protocol (Fig. 3). It can be observed (Fig. 3) that approximately 50% of the ZZ-cutinase-$(WP)_4$ did not bind to the column and eluted in the flow-through when an undiluted sample was applied on to the column. An explanation for this could be that demixing of the phases occurs upon loading an undiluted sample. In the following experiments 1 ml top phase was diluted with 250 μl of MilliQ water in order to avoid demixing. Consequently, the running buffer was also diluted to attain the same conductivity as the sample that in turn reduced the hydrophobic interaction between the matrix and the protein. Furthermore, the elution buffer was lowered to 5 mM sodium phosphate buffer (pH 7.2).

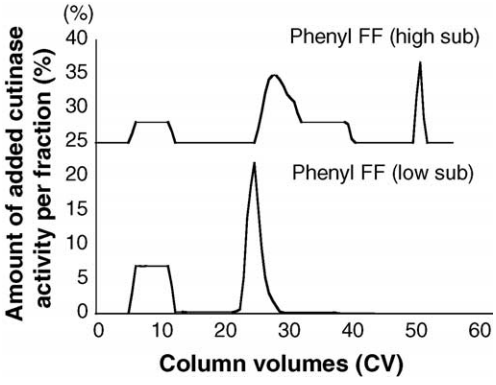

Fig. 3. Chromatogram showing the comparison of purified ZZ-cutinase-$(WP)_4$ on Phenyl FF (low sub) and Phenyl FF (high sub). Phenyl FF (high sub) has its zero value at 25% amount added activity of Phenyl (low sub).

3.3.4. Phenyl HP

Phenyl HP differs from the Phenyl FF matrices in ligand density and bead size [31]. The results (Table 6) showed that the mass balance (85%) for the Phenyl HP column did not close during the screening tests, which means that some activity of the ZZ-cutinase-$(WP)_4$ remained bound to the column. The lowering of the concentration of flow buffer and elution buffer (as discussed above in 3.3.3) resulted in an increased yield (87%) of ZZ-cutinase-$(WP)_4$ in the eluted peak compared to 78% during the screening tests (Table 6) The mass balance for the chromatography run was also increased from 85% to 91%. Furthermore, the eluted peak was less diluted than in the previous screening runs. Based on the increased yield, low dilution factor and purity (according to SDS–PAGE, results not shown) of the eluted peak from the Phenyl HP column compared to Phenyl FF (low sub) and Phenyl FF (high sub) it was concluded that Phenyl HP should be further optimised.

To enhance purity and yield of the eluted fraction from the Phenyl HP column it was decided to lower the flow rate during the sample load to the column from 0.5 to 0.25 ml/min in order to have a longer time for interaction of the proteins on the column. The flow rate during elution was also decreased from 1 ml/min to 0.5 ml/min. Furthermore, a linear gradient (20 CV) from 277.8 mM down to 50 mM running buffer was applied to the column during elution in order to achieve a higher purity of the sample. The ZZ-cutinase-$(WP)_4$ was, after the gradient, eluted in 5 mM phosphate buffer. This resulted in a fairly pure peak of the target protein with 83% yield. The goal was now to further increase the yield of the target protein in the eluted peak and to reduce the number of contaminants. A shallower gradient from 277.8 mM down to 20 mM running buffer was tested before 100% elution buffer (5 mM phophate buffer) was applied to the column. The flow rate during the gradient and elution was also lowered from 0.5 ml/min down to 0.25 ml/min. A silver stained SDS–PAGE (Fig. 4) gel showed that a very pure fraction

Table 7
Overall purification results for ZZ-cutinase-$(WP)_4$ in the two-step purification process where a top phase from an aqueous two-phase system is direct interfaced with hydrophobic interaction chromatography

Sample	Total activity U	Protein concentration (mg/ml)	Yield (%)	Purification factor
Homogenate	4522	45.1		
Top phase	4350	10.4	96	2.5
Start top phase HIC	824	8.32		
HIC	710	164.9	86	43.4
Overall purification			83	107

could be obtained with 87% yield of the target protein, ZZ-cutinase-$(WP)_4$. However, in a process scale application the continuous gradient should most likely be replaced by a step gradient procedure.

3.3.5. *Effect of tag on the target protein*

To evaluate the effect of the added hydrophobic $(WP)_4$ tag in both the aqueous two-phase extraction and HIC process steps, untagged ZZ-cutinase-wt was extracted in the optimised two-phase system (14% PEG 1500 and 10.5% K–Na phosphate salt). The top phase was then interfaced with the Phenyl HP column. The yield of ZZ-cutinase-wt in the two-phase extraction step was 56% compared to 96% yield for the ZZ-cutinase-$(WP)_4$. The lower yield in the aqueous two-phase system for ZZ-cutinase-wt step is in accordance with earlier published results [2]. One millilitre of top phase was then loaded on the Phenyl HP column, where the same optimised chromatography protocol as for the purification of ZZ-cutinase-$(WP)_4$ was used. The yield of ZZ-cutinase-wt was 71% compared to 87% for the tagged variant in the column step. The activity for ZZ-cutinase-wt could be detected in both the flow-through, during the gradient and in the eluted fractions. The overall yield in the two purification steps was 40% for the untagged protein and with a purification factor of 60. It can thus be concluded that the hydrophobic tag $(WP)_4$ increased the yield in the two-phase system as well as in the hydrophobic interaction chromatography compared with the untagged ZZ-cutinase-wt.

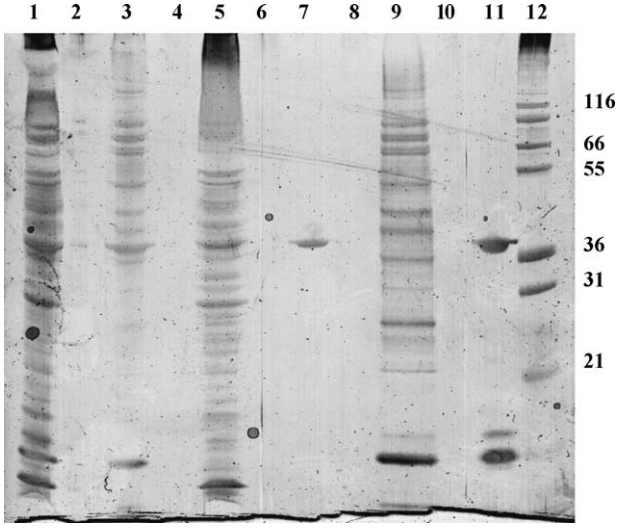

Fig. 4. A silver stained SDS–PAGE of purified ZZ-cutinase-$(WP)_4$ from the different purification steps. ZZ-cutinase-$(WP)_4$ can be visualised slightly above 36 kDa. Lane 1: homogenate, lane 2: –, lane 3: top phase, lane 4: –, lane 5: bottom phase, lane 6: –, lane 7: eluted ZZ-cutinase-$(WP)_4$, lane 8: –, lane 9: flow-through, lane 10: –, lane 11: IgG purified ZZ-cutinase-$(WP)_4$, lane 12: molecular weight marker.

3.4. *Evaluation of purity*

As can be visualized on the silver stained SDS–PAGE gel (Fig. 4), a 95% pure sample was achieved after the two combined methods. However, it was also interesting to monitor other contaminants than proteins. Thus, a reduction of 1000 EU of endotoxin in the final target protein sample was obtained compared to the starting sample. In addition, it was shown that most of the PEG was collected in the flow-through during the HIC run. No PEG (detection limit, 0.001 $g\,l^{-1}$) could be detected in the purified target protein sample.

3.5. *Conclusions*

This paper illustrates that the introduction of the tryptophan tag to the ZZ-cutinase protein can be used to facilitate the interfacing of aqueous two-phase extraction and hydrophobic interaction chromatography. A highly purified protein at high yield from an *E. coli* cell homogenate was achieved in the operation (Table 7). The ZZ-cutinase-$(WP)_4$ protein was purified to >95% purity according to silver stained SDS–PAGE gels with an overall yield of 83% during the two-step recovery process. Moreover, the final ZZ-cutinase-$(WP)_4$ sample contained no detectable PEG and the level of endotoxin was significantly reduced.

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References

[1] P.-Å. Albertsson, Partition of Cell Particles and Macromolecules, Wiley, New York, 1986.
[2] C. Kepka, E. Collet, J. Persson, A. Stahl, T. Lagerstedt, F. Tjerneld, A. Veide, J. Biotechnol. 103 (2003) 165.

[3] M.-R. Kula, K. Selber, in: S.W. Drew (Ed.), Enzyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation, Wiley, New York, 1999, p. 2179.
[4] K.H. Kroner, H. Schütte, M.-R. Kula, J. Chem. Technol. Biotechnol. 32 (1982) 130.
[5] N. Bandmann, E. Collet, J. Leijen, M. Uhlén, A. Veide, P.Å. Nygren, J. Biotechnol. 79 (2000) 161.
[6] K. Berggren, F. Tjerneld, A. Veide, Bioseparation 9 (2000) 69.
[7] S. Fexby, H. Ihre, J. Van Alstine, L. Bülow, J. Chromatogr. B 807 (2004) 25.
[8] C. Hassinen, K. Köhler, A. Veide, J. Chromatogr. A 668 (1994) 121.
[9] R.L. Fahrner, D.H. Whitney, M. Vanderlaan, G.S. Blank, Biotechnol. Appl. Biochem. 30 (Pt. 2) (1999) 121.
[10] V. Gaberc-Porekar, V. Menart, J. Biochem. Biophys. Methods 49 (2001) 335.
[11] M.T. Hearn, D. Acosta, J. Mol. Recognit. 14 (2001) 323.
[12] M. Manesse, R.C. Cox, B.C. Koops, H.M. Verheij, G.H. de Haas, M.R. Egmond, H.T.W.M. van der Hijden, J. de Vlieg, Biochemistry 34 (1995) 6400.
[13] B. Nilsson, T. Moks, B. Jansson, L. Abrahmsén, A. Elmblad, E. Holmgren, C. Henrichson, T.A. Jones, M. Uhlén, Protein Eng. 1 (1987) 107.
[14] S. Stahl, P.A. Nygren, Pathol. Biol. (Paris) 45 (1997) 66.
[15] F. Gharahdaghi, C.R. Weinberg, D.A. Meagher, B.S. Imai, S.M. Mische, Electrophoresis 20 (1999) 601.
[16] A. Shevchenko, O.N. Jensen, A.V. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, H. Boucherie, M. Mann, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 14440.
[17] B. Skoog, Vox Sang. 37 (1979) 345.
[18] K. Kohler, A. Veide, S.O. Enfors, Enzyme Microb. Technol. 13 (1991) 204.
[19] E. Collet, Licentiate Thesis at Department of Biotechnology, Royal Institute of Technology, Stockholm, 2003.
[20] W. Hummel, H. Schütte, M.-R. Kula, Eur. J. Appl. Microbiol. Biotechnol. 18 (1983) 75.
[21] H. Walter, G. Johansson, Methods Enzymol, Academic Press, New York, 1994.
[22] A. Veide, T. Lindbäck, S.-O. Enfors, Enzyme Microb. Technol. 6 (1984) 325.
[23] D. Forciniti, C.K. Hall, M.-R. Kula, Biotechnol. Bioeng. 38 (1991) 986.
[24] H.-O. Johansson, G. Karlström, F. Tjerneld, C.A. Haynes, J. Chromatogr. B 711 (1998) 3.
[25] P.A. Harris, G. Karlström, F. Tjerneld, Bioseparation 2 (1991) 237.
[26] J. Persson, L. Nyström, H. Ageland, F. Tjerneld, J. Chromatogr. B: Biomed. Sci. Appl. 711 (1998) 97.
[27] A. Veide, T. Lindbäck, S.-O. Enfors, Enzyme Microb. Technol. 11 (1989) 744.
[28] H. Hustedt, K.H. Kroner, M.-R. Kula, in: D. Fisher (Ed.), Partitioning in Aqueous Two-phase Systems: Theory, Methods, Uses and Applications to Biotechnology, Academic Press, New York, 1985, p. 529.
[29] J. Thommes, M. Halfar, H. Gieren, S. Curvers, R. Takors, R. Brunschier, M.R. Kula, Biotechnol. Prog. 17 (2001) 503.
[30] G. Sofer, L. Hagel, Handbook of Process Chromatography, Academic press, San Diego, 1997.
[31] Pharmacia Biotech, Hydrophobic Interaction Chromatography, Principles and Methods, 1993.